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APPENDIX

Mail Stop Amendment
Commissioner for Patents
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Alexandria, VA 22313-1450

Dear Sir:

The following attachments are provided:

(1) Telleman *et al.*, Biotechniques, 29(6):1240-1248 (2000);

(2) Demartis *et al.*, J. Mol. Biol., 286(2):617-633 (1999);

(3) Wang *et al.*, Mol. Cell. Biol., 21(14):4604-4613 (July 2001);

(4) Sinclair *et al.*, Biochem. Mol. Biol. Int., 31(5):911-922 (1993);

(5) Sarisky *et al.*, Biochem. Biophys. Res. Commun., 177(2):757-763, (1991);

(6) Li *et al.*, J. Cell. Biochem., 53(4):405-419, (1993);

(7) Werhahn et al., Plant Physiol., 125(2):943-954 (2001);

(8) Fountoulakis *et al.*, Anal. Biochem., 208(2):270-276 (1993);

(9) Morris *et al.*, Ther. Drug Monit., 14(3):226-233 (1992).

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

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Direct Immunoprecipitation of Antigen with Phage Displaying Immunoglobulin Fragment

BioTechniques 29:1240-1248 (December 2000)

ABSTRACT

Phage libraries may display hormones, receptors, antibody fragments, etc., by fusion to phage envelope proteins. This report describes the direct precipitation of phage-Fab-antigen complexes by polyethylene glycol precipitation, resulting in highly selective and efficient recovery of antigen from complex mixtures without nonspecific protein contamination. The method demonstrates efficiency and specific recovery of phage-Fab-antigen complexes from a background of a complex mixture of unrelated proteins as may occur in the analysis of biological specimens. This simple, fast, and effective method allows isolation and characterization of target antigens, with no need to further process Fab or sFv, and may reasonably be extended to isolate any interacting partner molecule for any displayed protein.

INTRODUCTION

Phage display technology is an in vitro selection technique in which a peptide or protein is genetically fused to a coat protein of a bacteriophage, resulting in display of the fused protein on the exterior of the phage virion. DNA encoding the fusion product resides within the virion. This linkage between the displayed protein (phenotype) and the encoding DNA (genotype) allows screening of vast numbers of variants of the protein by repeated rounds of selection and replication, a process termed panning (3,7,9,11). Although the first phage display libraries consisted of short peptides, libraries have become more sophisticated throughout the years, allowing fusion of hormones, receptors, antibody fragments, etc., to the phage envelope proteins (2,12). Phage display techniques are now widely used for the isolation of antigen-specific antibody fragments either as Fab (light chain plus Fd portion [V_H + C_H1] of the heavy chain) or sFv (light and heavy chain variable domains linked via a polypeptide) (4,8). Applications include the isolation of novel antibodies, in vitro improvement of affinity, and epitope discovery.

This report describes the direct precipitation of phage-Fab-antigen complexes by polyethylene glycol (PEG) precipitation at high efficiency without nonspecific protein contamination. This simple, fast, and effective method allows isolation and characterization of target antigens with no need to further process Fab or sFv.

MATERIALS AND METHODS

Phage Fab Clones

The anti-idiotype (anti-Id) phage Fab against mouse anti-Tac antibody (MAT) was cloned by combinatorial phage display techniques from B cells of a patient who was treated with MAT (6). An anti-tetanus toxoid (anti-TT) phage Fab was used as a nonspecific control (gift of D. Burton, Scripps Research Institute, La Jolla, CA).

Phage Culture (1)

Phage stock (20 μL) was added to a fresh culture of 10 mL *E. coli* strain XL1 Blue and incubated at room temperature for 15 min. Prewarmed superbroth (SB) medium (10 mL), containing 20 μg/mL ampicillin and 10 μg/mL tetracycline, was added and incubated for 1 h at 37°C with shaking. The 12-mL culture was added to 100 mL SB, containing 50 μg/mL ampicillin and 10 μg/mL tetracycline, and incubated for 1 h at 37°C with shaking. Helper phage VCSM13 (1 mL) (total of 10^{12} pfu) was added to the culture and incubated for 2 h at 37°C with shaking. After adding kanamycin to a concentration of 70 μg/mL, incubation was continued overnight at 37°C with shaking. Cells were spun down at 4000× *g* for 15 min at 4°C. Twenty milliliters of 20% (w/v) PEG-8000/2.5 M NaCl were added to the supernatant and incubated on ice for 30 min. The phage precipitate was collected by centrifugation at 8000× *g* for 30 min at 4°C. The pellet was resuspended in 1.5 mL PBS/1% BSA. Debris was removed by centrifugation for 13 000× *g* at 4°C for 5 min. The supernatant was titered and stored at 4°C.

Preparation of Membrane Proteins (10)

Human HTB24 cells (5 × 10^7) were washed twice with ice-cold PBS and scraped from the culture plate in a total volume of 5 mL ice-cold PBS. The cells were precipitated at 1000× g for 5 min at 4°C. The cell pellet was resuspended in 2 mL swelling buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM PMSF, 0.15 U/μL aprotinin, 20 μM leupeptin, 1% Triton® X-100) and incubated on ice for 15 min. The suspension was subjected to 30 strokes in a glass Dounce homogenizer. Nuclei were removed by centrifugation at 1500× *g* for 5 min at 4°C. Membrane proteins were precipitated by centrifugation at 12 000× *g* for 30 min at 4°C. The membrane proteins were dissolved in 0.5 mL solubilization buffer (swelling buffer plus 0.1% SDS), aliquoted, and stored at -80°C.

Protein Labeling

MAT was labeled with ^{131}I (1 μCi/μg). Human immunoglobulin (huIgG) and mouse serum albumin (MSA) were labeled with ^{125}I (5 μCi/μg and 1.6 μCi/μg, respectively). Human membrane proteins were also labeled with ^{125}I (1.6 μCi/μg). Proteins were radiolabeled with Iodobeads (Pierce Chemical, Rockford, IL, USA) and separated from free iodide by size exclusion on a Sephadex® PD10 G-25 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Radioactivity was determined in a Model 5500 dual channel gamma counter (Beckman Coulter, Fullerton, CA, USA), with corrections applied for radioactive crossover and decay. Labeled proteins were stored at 4°C.

Immunoprecipitation

Phage stocks and labeled proteins were prespun at 13 000× *g* for 30 min at 4°C to remove any precipitate. Phage (10^{12} pfu) were incubated with labeled

protein for 2 h on ice in a total volume of 300 µL PBS/0.1% BSA. Phage were precipitated by adding 60 µL 20% (w/v) PEG-8000/2.5 M NaCl, incubation on ice for 30 min, and centrifugation at 13 000× *g* for 30 min at 4°C. The amount of label in the supernatant and the pellet was determined in a dual channel gamma counter.

Gel Electrophoresis

^{131}I-labeled MAT (10, 50 and 100 ng) were incubated with 10^{12} pfu anti-Id phage and immunoprecipitated as above. An incubation of 100 ng ^{131}I-labeled MAT with 10^{12} pfu TT served as a negative control. To all incubations, 2000 ng of a ^{125}I-labeled mem-

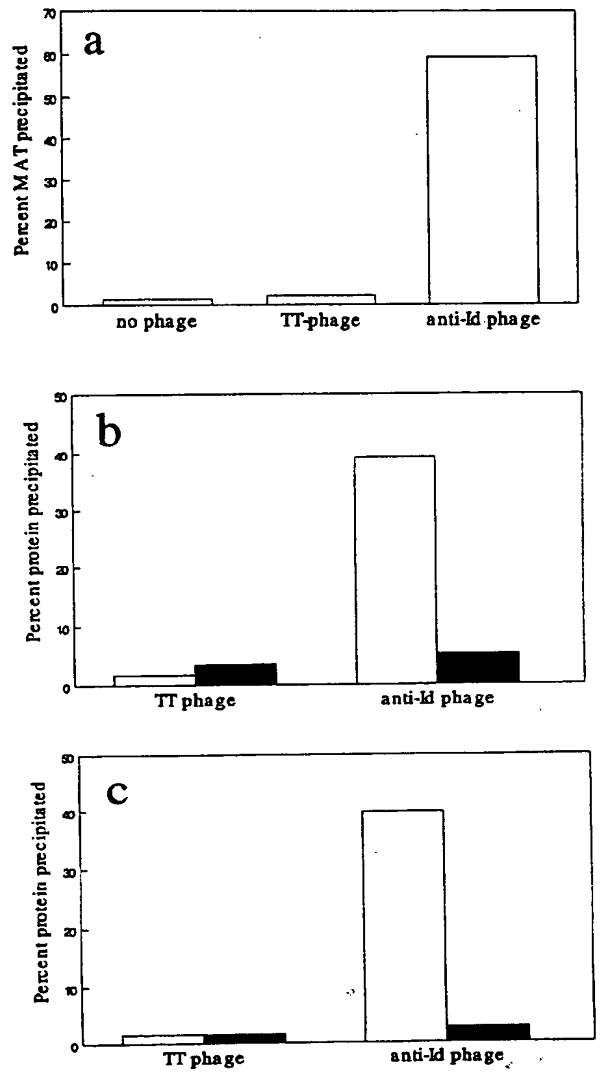

Figure 1. Specific immunoprecipitation of antigen by phage-Fab. (a) Precipitation of ^{131}I-labeled MAT (□) after incubation with no phage, nonspecific TT phage, or specific anti-Id phage. (b) Precipitation of ^{131}I-labeled MAT (□), and ^{125}I-labeled MSA (■) after incubation with TT phage or anti-Id phage. (c) Precipitation of ^{131}I-labeled MAT (□) and ^{125}I-labeled huIgG (■) after incubation with TT phage or anti-Id phage. In repeat experiments, errors were ± 1%–2% for the low recoveries and ± 5% for the high recoveries.

brane protein preparation (1.6 µCi/µg) were added as nonspecific protein background. The phage pellets were resuspended in 50 µL PBS/0.1% BSA and centrifuged again at 13 000× *g* for 30 min at 4°C to remove any precipitate. Ten microliters of the supernatant, containing phage-Fab-protein complex, were mixed with 5 µL loading buffer containing β-mercaptoethanol as the reducing agent, loaded on a SDS/polyacrylamide gradient gel, and run for 2 h at 100 V. The gel was transfered to filter paper, dried, and autoradiographed overnight using a single screen.

RESULTS

Bacteriophage can be precipitated by PEG at high salt concentrations, a method that is widely used to concentrate bacteriophage. Under these same conditions, soluble proteins are virtually

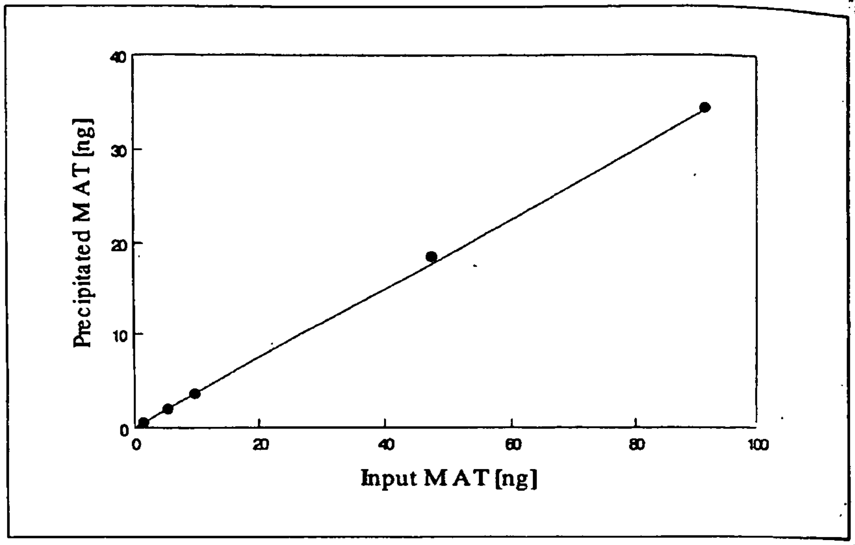

Figure 2. Efficient immunoprecipitation with anti-Id phage. Increasing amounts of ^{131}I-labeled MAT were incubated with 10^{12} pfu specific anti-Id phage in the presence of 100 ng ^{125}I-labeled nonspecific huIgG. The amount of antigen (MAT) precipitated increased linearly with increasing input amounts of MAT antigen for a constant efficiency of precipitation (35%–40%).

not precipitated. We show here that the same technique can be applied to the precipitation of phage-Fab-antigen complexes. The antigen may subsequently be released from the complex with low pH conditions (1,11). This technique mimics the widely used immunoprecipitation of antibody-antigen complexes by protein A. The method achieves isolation of antigen with no need to further manipulate phage clones.

The method is demonstrated with a model antigen-antibody pair. The antigen for this test is an antibody (MAT) for which a human anti-Id antibody was cloned as a phage Fab. Nonspecific phage Fab was specific to TT.

Immunoprecipitation with Phage

To determine the feasibility and specificity of immunoprecipitation of MAT with anti-Id phage, 100 ng ^{131}I-labeled MAT (1 µCi/µg) was incubated with 10^{12} pfu anti-Id phage or TT phage. The results show that MAT is precipitated with anti-Id phage only, but not with TT phage or by PEG/NaCl without specific phage (Figure 1a). Although the antigen in this case (MAT) is a large protein (150 kDa), it is not nonspecifically precipitated by PEG under the conditions, but only in the presence of anti-Id phage.

In co-precipitation experiments, ^{131}I-labeled MAT was mixed with 500 ng ^{125}I-labeled MSA (Figure 1b) or 100 ng ^{125}I-labeled huIgG (Figure 1c). In each case, only MAT was precipitated. Finally, the efficiency of immunoprecipitation of MAT with anti-Id phage was determined by incubating increasing amounts of ^{131}I-labeled MAT (1 ng to 100 ng) with 10^{12} pfu anti-Id phage in the presence of 100 ng ^{125}I-labeled huIgG (Figure 2). The amount of MAT that is precipitated increased linearly with increasing input amounts of MAT, with a relatively constant fractional antigen recovery (35%–40%) over the full range.

Gel Electrophoresis

To demonstate further the specificity of the immunoprecipitation, the presence of protein of the appropriate size in the immunoprecipitate was examined by gel electrophoresis and autoradiography. Increasing quantities (10, 50, and 100 ng) of ^{131}I-labeled MAT (1 µCi/µg) were incubated with 10^{12} pfu anti-Id phage in the presence of 2000 ng ^{125}I-labeled total nonspecific human membrane proteins (1.6 µCi/µg). Proteins eluted from the PEG precipitate by PBS were separated on a gradient SDS/polyacrylamide gel under reducing conditions and visualized by autoradiography (Figure 3). The radioactive heavy chain (50 kDa) and light chain (25 kDa) of MAT, which was released from the PEG-precipitated phage-Fab-MAT complex, are clearly seen with increasing intensities, whereas no bands of the constant amount of excess nonspecific membrane proteins were evident. (At the lowest MAT concentration, the nonspecific protein is 200-fold in mass excess, or 50- to 100-fold excess in effective radioactivity when allowing for the 4- to 5-fold higher film capture efficiency of ^{131}I; data not shown.) No radioactive protein was precipitated with TT phage.

DISCUSSION

After the introduction of phage display technology, it has quickly become an important tool for the isolation of Fab or sFv fragments with high affinities for particular antigens. In this paper,

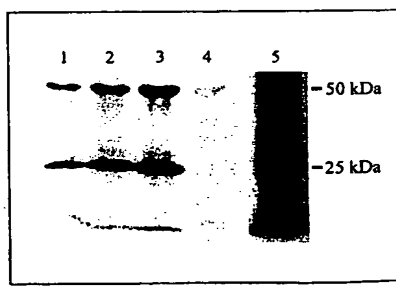

Figure 3. Gel electrophoresis of immunoprecipitated radioactive protein. To all samples, 2000 ng of a ^{125}I-labeled membrane protein preparation was added as a nonspecific background. Immunoprecipitated proteins were resuspended and run on reducing SDS/polyacrylamide gels. Lane 1, 10 ng; lane 2, 50 ng; and lane 3, 100 ng of ^{131}I-labeled MAT precipitated with 10^{12} anti-Id phage. Lane 4, 100 ng ^{131}I-labeled MAT precipitated with 10^{12} TT phage. Lane 5, pattern of added nonspecific labeled membrane proteins before immunoprecipitation. The light chain (25 kDa) and heavy chain (50 kDa) of ^{131}I-labeled MAT are clearly visible in lanes 1–3 without significant contaminating nonspecific proteins.

we demonstrate the possibility to isolate antigen directly by PEG precipitation with phage displaying antigen-specific Fab. The method is based on the precipitation of phage by PEG, a technique that is frequently used to concentrate bacteriophage. The results demonstrated here with a model phage Fab-antigen pair show that direct PEG precipitation of phage-Fab-antigen complexes is both specific and highly efficient. Typically, free antigen can be obtained from the recovered phage-Fab-antigen complexes by dissociation at pH 2.0–3.0 (1,11) and then separated by physical methods for further analysis.

The highest concentration of MAT tested (100 ng) corresponds to approximately 4×10^{11} molecules (0.6 pmol), which is less than the total phage used in these precipitations (10^{12}, 1.6 pmol); therefore, a phage excess was maintained throughout. The observed constant fractional precipitation of antigen (MAT; 35%–40%) in the presence of excess antibody (phage-Fab) allows derivation of the K_d, the concentration of free antibody at which 50% of antigen is bound. Phage (10^{12}) per 0.3 mL in our assays is 6 nM in phage. From the 35%–40% MAT precipitation, an effective K_d of approximately 15 nM or K_a of 7×10^7/M may be estimated. The K_a of soluble anti-Id for immobilized MAT was previously estimated as approximately 2×10^7/M (6), which is of the same order of magnitude as the present estimate, albeit modestly lower. However, a direct comparison of these numbers requires an assumption that there is an average of one Fab per phage; less than one or more than one Fab per phage will mean the affinity is an underestimate or overestimate, respectively. From the linearity of Figure 2 up to 100 ng MAT, it can be shown that the number of Fab per phage Fab cannot be much less than unity, but MAT saturation data were not obtained from which to derive a specific estimate. Thus, the higher phage Fab affinity may reasonably be attributed to more than one Fab per phage or to technical differences in the assay methods.

By elementary principles of mass action, other phage-Fab with higher or lower antigen affinity than the present example may be predicted to be correspondingly more or less efficient at antigen recovery, in which lower antigen recoveries may be expected to be improved by higher concentrations of phage. This validates the applicability of basic principles of biochemical affinity in this precipitation method for antigen capture. As a modification of this approach, other versions of phage display, which use coat protein VIII (cpVIII) with multivalent display (100s per phage) versus the nominal monovalent display of the cpIII-modified phage of our example, have the potential to substantially increase the yield per phage of antigen or ligand on immunoprecipitation and would be expected to be equally well adapted to this method.

The method described here provides an alternative to conventional immunoprecipitation of antigen with antibody or antibody fragments that has found general application in the isolation and characterization of antibodies and antigens (5). Although this paper demonstrates the technique by precipitating antigen with specific phage displaying the antibody Fab fragment, it should be generally applicable to all situations in which affinity exists between a protein displayed on the phage and a target molecule. This opens the possibility to study interactions between a variety of molecules, something that cannot be achieved with conventional immunoprecipitation. Circumventing the need to purify protein that is displayed on phage provides a further significant benefit over traditional techniques and opens up the way to faster and more efficient antigen-antibody or ligand-receptor screening.

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